AD-A271 643

2

AD_____

GRANT NO: DAMD17-89-Z-9016

TITLE: IMMUNOTOXICOLOGICAL ASSAYS USING THE JAPANESE MEDAKA

PRINCIPAL INVESTIGATOR: Robert S. Anderson

CONTRACTING ORGANIZATION: University of Maryland, CEES
Chesapeake Biological Laboratory
P.O. Box 38
Solomons, Maryland 20688-0038

DTIC
ELECTE
OCT 29 1993
S A D

REPORT DATE: July 31, 1992

TYPE OF REPORT: Annual/Final Report

PREPARED FOR: U.S. Army Medical Research and
Development Command, Fort Detrick
Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

93-26237

64pf

93 10 28 016

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Distribution for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of Maryland System CEES; 6b. OFFICE SYMBOL (If applicable): N/A	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Chesapeake Biological Laboratory, P.O. Box 38, Solomons, Maryland 20688-0038	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: Grant No. DAMD17-89-Z-9016
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21702-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62720A	3E1-62720A835	00	DA317774

11. TITLE (Include Security Classification)

Immunotoxicological Assays Using the Japanese Medaka

12. PERSONAL AUTHOR(S)

Robert S. Anderson, Ph.D. and Lisa L. Brubacher, M.S.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual/Final	FROM 12/15/88 TO 6/14/92	July 31, 1992	58

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	immunology, immunotoxicology, fish, Japanese medaka, macrophages, reactive oxygen intermediates, RA3
06	11		
06	05		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Fish phagocytic blood cells provide the first line of defense against pathogenic microorganisms that cause infectious diseases. The blood cells engulf bacteria and kill them via cytotoxic mechanisms mediated by the production of various oxyradicals. Exposure of fish to sublethal concentrations of environmental contaminants reportedly causes reduced resistance to disease via immunological suppression. The goal of this study was to determine the effects of xenobiotics on the immune systems of a representative bivalve mollusk (the Eastern oyster) and a small aquarium fish (the medaka) by quantifying changes in oxyradical production by macrophages. In this way, it was possible to provide evidence of immunotoxicity of several classes of common aquatic pollutants such as heavy metals and halogenated phenolic compounds. For example, the priority pollutant pentachlorophenol, an environmentally ubiquitous chemical, was shown to markedly reduce oxyradical production by the blood cells of both oysters and fish. Some of the tests developed during this study, especially luminol-augmented chemiluminescence, proved to be very sensitive and may find application in monitoring chronic aquatic pollution.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mrs. Virginia M. Miller	(301) 619-7328	SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

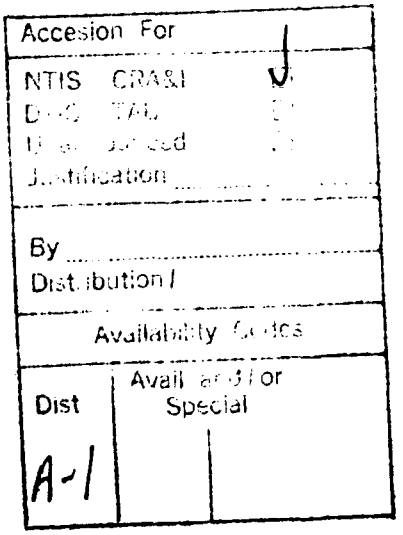
___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

RSA Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

RSA In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

___ For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

___ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.



Robert S. Anderson June 22, 1993

P. I. Signature Date

LIST OF TABLES

LIST OF FIGURES

TABLE OF CONTENTS

INTRODUCTION

The major objective of this study was to quantify immunotoxic effects of xenobiotics on the Japanese medaka (*Oryzias latipis*). During the first year, while details of medaka husbandry were being worked out, some initial immunotoxicological studies were carried out on the Eastern oyster (*Crassostrea virginica*). Oysters were routinely maintained in flow-through estuarine aquaria in our controlled environmental laboratory. Initial attempts to obtain peripheral blood and peritoneal macrophages from medaka were unsuccessful, in that insufficient cells were obtained to carry out the desired immunoassays. However, oyster hemocytes (macrophages) were abundant and were used to estimate the potential difficulty of adapting mammalian immunoassays for aquatic species. When it was determined that phagocytes in adequate numbers could be obtained from medaka pronephros, the oyster hemocyte studies were phased out and the effort was concentrated on medaka macrophages. The macrophage is particularly valuable in immunotoxicology testing because it not only participates directly in the destruction of pathogenic microbes, but also modulates lymphocytes via cytokines and is involved in antigen processing and presentation. This study has concentrated on macrophage bactericidal activity (as measured by the chemically-induced modulation of macrophage-generated reactive oxygen intermediates); macrophage bactericidal activity has been validated as a sensitive and predictive immunoassay in mice and rats (National Research Council, 1992).

It has been known for some time that the process of phagocytosis by mammalian polymorphonuclear leukocytes (PMNs) and macrophages is accompanied by an abrupt increase in oxygen uptake, followed by the production of cytotoxic reactive oxygen intermediates (ROI) (See reviews by Badwey and Karnovsky, 1980; Beaman and Beaman, 1984; Fridovich, 1988).

It is accepted that the first reaction in the respiratory burst is the one electron reduction of oxygen to superoxide (O_2^-) catalyzed by NADPH oxidase associated with the phagocyte membrane. Superoxide anions are converted to hydrogen peroxide (H_2O_2) by the cytoplasmic enzyme superoxide dismutase (SOD) (Fridovich, 1988). Superoxide and, to a greater extent, H_2O_2 are highly reactive and toxic ROIs; H_2O_2 in conjunction with myeloperoxidase (MPO) and a halide forms the basis of a potent leukocyte antibacterial system (Klebanoff, 1968). Other toxic ROIs can also be generated, such as hydroxyl radicals (.OH) and singlet oxygen ($^1O_2$). Singly or collectively, these ROIs can participate in the cell-mediated destruction of bacteria, fungi, and protozoa. The phagocytes have detoxification enzymes to protect themselves against auto-oxidative damage, these include SOD, glutathione peroxidase, catalase, certain vitamins that are radical scavengers, etc.

Hemocytes from several molluscan species seem to undergo a typical respiratory burst with concomitant ROI production. Early attempts to measure increased oxygen uptake and/or MPO-halide-H_2O_2 system activity by phagocytosing clam hemocytes were unsuccessful (Cheng, 1976). However in 1985, O_2^- production in stimulated snail hemocytes was reported by Dikkeboom et al (1985) and H_2O_2 production by resting and stimulated scallop hemocytes was shown by Nakamura et al. (1985). Papers on ROI production by molluscan hemocytes have proliferated since that time. In a 1991 review of this subject, Adema et al. (1991) cite 14 references on ROI activity in 13 species of gastropods and bivalves. In these papers O_2^- was measured by reduction of NBT or cytochrome c, and H_2O_2 was measured by colorimetric assays (diaminobenzidine or phenol red oxidation) or in fluorescence assays (homovanillic acid). Cellular chemiluminescence (CL), in the presence of signal-amplifying probes, was also used as

an indicator of ROI production. The CL method does not yield quantitative information on particular ROI species; the contribution of O_2^- or H_2O_2 to the CL signal can be estimated by the use of appropriate enzymatic inhibitors. It is widely accepted that the CL response of phagocytes is correlated with bactericidal activity associated with the respiratory burst (Welsh, 1980; Horan et al., 1982), and the method has been specifically recommended for use with fish and shellfish. The detection of luminol-augmented CL using stimulated hemocytes has been used to estimate ROI production in several bivalves such as *Crassostrea virginica*, (Larson et al., 1989) *Crassostrea gigas*, and *Ostrea edulis* (Bachère et al., 1991), and *Pectin maximus* (LeGall et al., 1991). Likewise, CL assays indicate ROI induction after stimulation of hemocytes from gastropods including *Lymnaea stagnalis* (Adema et al., 1991), *Helix aspersa* (Dikkeboom et al., 1988), and *Achatina achatina* (Adema et al, 1992).

The CL response of oyster (*Crassostrea virginica*) blood cells either exposed in vitro or collected from oysters after various periods of in vivo exposure to selected heavy metals, pesticides or other organic compounds, was measured by Larson et al (1989). They found that copper in both in vivo and in vitro studies, was the most effective agent tested with regard to ability to depress the CL response, although most of the compounds appeared to suppress CL, particularly at high exposure levels. Certain compounds, such as cadmium, aluminum, zinc, dieldrin and naphthalene, apparently caused increased CL at low levels, but this effect was usually reversed at higher concentrations.

Fisher et al. (1990) reported that in vitro tributyltin (TBT) treatment of hemocytes from *C. virginica* or *C. gigas* produced slight stimulation at low concentrations (0.4 ppb), followed by dose-dependent suppression of CL at higher concentrations. The suppressive TBT

concentrations (40-400 ppb) exceeded those found in most environmental samples; nonetheless, hemocytes of field exposed oysters are probably exposed to high TBT levels as a result of bioaccumulation.

Work in this laboratory has demonstrated the immunosuppressive effect of cadmium on oyster hemocyte CL (Anderson et al., 1992). When the cells were exposed in vitro to sublethal cadmium levels, a dose-dependent inhibition of ROI production was consistently recorded. Phagocytically-induced CL was inhibited by ≥2ppm Cd, and this inhibition was shown to be a function of intracellular cadmium concentration. Similar chemically-induced inhibition of luminol-augmented CL responses of oyster hemocytes have also been produced by exposure to particulate brass, copper, and pentachlorophenol (Roszell and Anderson, 1992; Anderson et al., 1992; 1993). Such evidence implicates these xenobiotics as potential immunotoxicants, by extension of the criteria already developed for mammals (Tam and Hinsdill, 1990).

Aerobic killing by mammalian phagocytes is mediated by ROIs produced subsequent to the reduction of molecular oxygen to O_2^- via a membrane-associated NADPH oxidase. Its components form an electron transport chain with a flavoprotein component accepting electrons from NADPH, transferring them to O_2 via cytochrome b. Secombes et al. (1992) presented evidence that fish macrophages also have a cytochrome b component of the NADPH oxidase similar to that typical of mammals. Furthermore, specific inhibitors of the flavoprotein and cytochrome b_{-245} components of NADPH oxidase were both shown to inhibit the respiratory burst activity of trout macrophages.

As previously mentioned, luminol-enhanced blood cell chemiluminescence (CL) is one of the most frequently used techniques to quantify ROI production. This method has been

applied to blood cells from various species of fish. The CL response of pronephros cells from *Morone saxatilis* was measured after exposure to bacterial pathogens (Stave et al., 1983). Various phagocyte:bacteria ratios were employed, each producing somewhat different CL responses with regard to the magnitude of the activity and the individual kinetics. In addition to bacteria, both particulate (zymosan) and soluble (phorbol myristate acetate) classical activators of CL by mammalian leukocytes were shown to be effective with fish cells (Stave et al., 1984). Prior incubation of test particulates with various serum preparations has been generally found to enhance, or opsonize, phagocytic activity of fish leukocytes based on measurements of CL activity (Scott and Klesius, 1981). For example, channel catfish peripheral blood leukocytes, when mixed with *Edwardsiella ictaluri* previously incubated with autologous immune serum, produced an elevated CL response, as compared to that elicited by untreated on nonimmune serum treated *E. ictaluri* (Scott et al., 1985). The opsonic activity of immune serum was attributed to the presence of both specific antibodies and complement.

Preliminary studies suggest that fish phagocytes show significantly reduced CL activity following in vitro or in vivo exposure to xenobiotics. Elsasser et al. (1986) studied the CL response of rainbow trout pronephros phagocytes to *Staphylococcus aureus* after exposure to various metals. They found that copper was particularly effective in reducing macrophage CL, which might explain the observations that copper exposure can predispose fish to infection when challenged with viral (Hetrick et al., 1979) or bacterial (Knittel, 1981) pathogens. Warinner et al. (1988) examined the response of kidney phagocytes from control and pollutant-exposed fish. The zymosan-elicited CL response in spot (*Leiostomus xanthrus*) collected from the PAH-polluted Elizabeth River was negligible compared to the response from spot from the York River (a more

pristine site). This same suppression of phagocyte CL could be induced under laboratory conditions by exposing spot to contaminated Elizabeth River sediments. Exposure of dab (*Limanda limanda*) to sewage sludge also inhibited the respiratory burst, as indicated by reduced O_2^- production (Secombes et al., 1991).

The inhibitory effects of in vitro exposure to tributyltin (TBT) on CL of phagocytes from kidneys of three species of estuarine fish were studied by Wishkovsky et al. (1989). Other investigators also found that similar concentrations of TBT (~500 μg/l) inhibited CL, but a lower dose (50 μg/l) was stimulatory when oyster toadfish macrophages were exposed in vitro (Rice and Weeks, 1989). The opposing effects were attributed to changes in calcium flux across the hemocyte membrane induced by different concentrations of TBT. Peritoneal macrophages removed after in vivo exposure of the fish to TBT showed a dose-dependent suppression of CL (Rice and Weeks, 1990).

Exposure of medaka (*Oryzias latipes*) pronephros phagocytic blood cells to a ubiquitous environmental contaminant, pentachlorophenol (PCP), produced a dose-dependent inhibition of phagocytically-induced CL (Anderson and Brubacher, 1992; 1993). PCP also produced decreased resistance to bacterial infection in the clam, *Mercenaria mercenaria*, as a consequence of impaired hemocyte-mediated antibacterial capacity (Anderson et al., 1981; Anderson, 1988). One could reasonably speculate that this was due to decreased ROI production. With regard to the effects of analytical grade PCP on phagocyte function in fish and bivalves, it is interesting to note that 2,4-dinitrophenol, another uncoupler of oxidative phosphorylation, will inhibit superoxide release and CL by rat and rabbit alveolar macrophages (Miles et al., 1977; Castranova et al., 1987).

METHODS

1. Preparation of Macrophages (Medaka)

Medaka were stunned by a rapid blow to the head and immediately decapitated. Head kidney (pronephros) tissue was collected and pooled in 2 ml of augmented Leibovitz medium (L15, see chemiluminescence protocol for formulation). Tissue was transferred in a total of 4 ml aug. L15 to a Dounce tissue grinder on ice and gently disrupted by 20 strokes with pestle A (~9-17μm clearance) and 15 strokes with pestle B (~3-9 μm clearance). Cell clumps and other material were allowed to settle for 2-3 min and the overlying cell suspension transferred to a sterile conical tube. The cell population appeared to be quite homogeneous and was >96% positive for nonspecific esterase (a marker enzyme for macrophages); therefore, no further attempts were made to fractionate it by density gradient centrifugation or otherwise enrich it for macrophages. The cell suspension was introduced to both chambers of a standard hemacytometer, counted, and finally adjusted to $2X10^6$/ml.

2. Luminol-augmented Chemiluminescence (CL)

a. CL by Oyster Macrophages

1. Hemolymph (~6 ml) from 4-7 oysters was withdrawn from the adductor muscle hemolymph sinus and held in a plastic tube on ice to minimize cell clumping. One ml hemolymph aliquots were introduced into pony liquid scintillation counter vials. Half ml aliquots from each pool were saved for total hemocyte count determinations.

2. Oyster serum contains proteins that can bind metals, thereby reducing their availability (and toxicity) to the hemocytes; therefore, the aliquots were centrifuged (525xg, 10

min, 21°C), the serum removed, and the cell resuspended in sterile (filtered, 0.45 μm pores) ambient estuarine water (18 ppt) containing 5.5 mM dextrose; this was termed "FA".

3. Exposure procedure: hemocytes in 1 ml FA, 0.3 ml toxicant solution, 20μl antibiotic/antimycotic solution (200 U penicillin G, 0.2 mg streptomycin, 0.5 μg amphotericin B), were incubated in the dark for 3h at 21°C. Controls: hemocytes in 1.3 ml FA, 20 μl antibiotic/antimycotic solution. Incubated as per exposed samples.

4. After incubation, one ml 250 μM luminol was added and the resting (unstimulated) CL was recorded for 10 min in a Packard-Tri-carb 1900 CA liquid scintillation counter with single photon monitor. Then 0.2 ml of heat-killed yeast suspension (10 mg bakers yeast/ml FA) was added to each tube and the phagocytically-induced CL recorded for at least one hour.

b. Luminol-augmented Chemiluminescence (Medaka Macrophages)

1. Place 1.0 ml aliquots of cell suspension (2 X 10^6 macrophages/ml aug. L15) into small (pony) scintillation vials. This and all following steps are carried out with dark-adapted (>24 hr) vials and reagents, in a dark room under dim red illumination.

2. Tubes are centrifuged (10 min, 21°C, 300 xg) and supernatants replaced by 1.0 ml aug. L15 (controls) or 1.0 mil aug. L15 containing toxicant (experimentals). Resuspend cells and incubate 1-20 hr in the dark (21°C).

3. Centrifuge control and experimental tubes (10 min., 21°C, 300xg), discard all supernatants (to remove toxicant) and resuspend in aug. L15. Carry out viability studies (trypan blue exclusion) on one drop of each cell suspension.

4. Add 50μl lmM luminol to each vial, mix gently and place in Packard Tri-carb 1900 CA liquid scintillation analyzer operated in single photon counting mode, 60% voltage to photomultiplier tube, static suppression off. Count each sample for 0.33 min for a least 3-4 cycles to establish background (resting) CL.

5. Add 0.1 ml yeast solution to each vial, count each vial in scintillation analyzer as above; 0.33 min counts are made sequentially for at least 2 hr to establish the kinetics of the phagocytically-induced CL response.

Solutions:

Augmented L15: 5% fetal calf serum, 3% antibiotic-antimycotic solution, in Leibovitz L15 medium (Sigma Chemical Co.). Filter sterilize (0.2μm filter) and store at 4°C.

Yeast: 2g dehydrated bakers yeast is resuspended in 50 ml sterile phosphate buffered saline (PBS, Sigma Chemical Co.) and autoclaved. Yeast is washed 2x in sterile PBS, resuspended in 50 ml sterile PBS, divided into 1 ml aliquots and stored at -20°C. Prior to use thaw aliquot and determine the numbers of yeast cells present/ml with a hemacytometer. Adjust yeast to 4 x 10^7/0.1 ml PBS. Addition of 0.1 ml yeast solution to macrophage suspension should give a ratio of 20 yeast cells/macrophage.

Luminol: Stock soln: 0.014g luminol, 0.618g boric acid, 0.780g KOH in 10 ml sterile water, equals 7.9 mM (method of Scott and Klesius, 1981). Stock solution can be kept at -20°C for one week. Working stock (1 mM) is made by diluting 1 ml of stock (7.9

mM) in 6.9 ml Hanks balanced salt solution. Working stock should be added to CL tubes within 2 hrs of preparation and kept in the dark.

3. Extracellular Superoxide Anion Generation by Medaka Macrophages via Ferricytochrome c

a. Place 1.0 ml aliquots of cell suspension ($2x10^6$ macrophages/ml aug. PBS) into conical plastic centrifuge tubes, centrifuge (10 min., 21°C, 300 xg). Replace supernatant with 1.0 ml aug. PBS (controls) or 1.0 ml aug. PBS containing toxicant (experimentals). Resuspend cells and incubate 1-20 hrs (21°C).

b. Centrifuge (10 min., 21°C, 300 xg) and discard supernatant (toxicant). Resuspend cells in 1.2 ml cytochrome c (80μM) for "unstimulated" sample, resuspend second, identical aliquot in 1.2 ml cytochrome c (80μM) containing 10μM phorbol myristate acetate (PMA) for the "stimulated" sample. To an empty conical tube add 1.2 ml cytochrome c (80μM) for the "cell-fee control" sample.

c. Incubate tubes at 21°C, with gentle agitation every 15 min., for 60 min. Centrifuge (10 min., 21°C, 525xg).

e. Read O.D. of supernatants at 550 nm vs. "cell-free control" tubes.

f. Calculate O_2^- generated using extinction coefficient for reduced cytochrome c.

$$\Delta E_{550nm} = 21x10^3\ M^{-1}cm^{-1}$$

Calculate % modulation of O_2^- produced by exposure to toxicant concentrations.

Solutions:

Augmented PBS: 5% fetal calf serum, 3% antibiotic-antimycotic solution, 0.1% dextrose in phosphate buffered saline (PBS), all from Sigma Chemical Co. Filter, sterilize (0.2μm) and store at 4°C.

Cytochrome c solution: 9.9 mg ferricytochrome c (Sigma #C-2506) from horse heart (type 3) in 10 ml aug. PBS, (80μM). Use within 1 hr.

PMA: 2mM stock solution: 10mg PMA (phorbol 12-myristate 13 acetate, Sigma #P-8139) in 8.106 ml dimethylsulfoxide (DMSO). Freeze 100μl aliquots, store at -20°C in dark. One hour prior to use: dilute 2mM stock PMA in 80μM cytochrome c to yield 10nM PMA in cytochrome c solution.

4. Hydrogen Peroxide Assay (Medaka Macrophages)

a. Introduce 1 ml aliquots of macrophage suspension (2 x 10^6 cells) to sterile plastic test tubes; 4 tubes will be required for each toxicant dilution tested. Centrifuge tubes (10 min., 21°C, 250 xg), discard supernatants, and resuspend cells in xenobiotic solutions in 1 ml aug. PBS (experimentals) or 1 ml. aug. PBS (controls). Incubate 1-20 hrs at 21°C.

b. Centrifuge tubes (10 min., 21°C, 250 xg) replace supernatants with 1.2 ml phenol red solution (PRS) without PMA (unstimulated samples) or 1.2 ml PRS containing 10nM PMA (phorbol myristate acetate-stimulated samples). Incubate tubes 60 min. at 21°C, with gentle agitation every 15 min.

c. Centrifuge tubes (10 min., 21°C, 525 xg) and transfer supernatants to tubes containing 10μl IN NaOH. Read O.D. at 610nm against 1.0 ml PRS plus 10 μl IN NaOH.

d. Calculate H_2O_2 generated by unstimulated and stimulated macrophages using standard curve.

e. Standard curve: Prepare ~0.01M H_2O_2 by diluting 1.02 ml 30% H_2O_2 to 1000 ml with distilled water; actual concentration is determined by the extinction coefficient $\Delta E_{230nm} = 81 \times M^{-1} cm^{-1}$. Prepare a series of dilutions of this stock in distilled water in the range 0.1 - 100μM H_2O_2. Add 10μl of each dilution to 1 ml PRS, incubate 5 min. at 21°C. Add 10μl IN NaOH to each sample and read against a mixture of 1 ml PRS + 10 μl H_2O + 10 μl IN NaOH at 610 nm. Construct standard curve.

Solutions

Aug. PBS: 5% fetal calf serum, 3% antibiotic-antimycotic solution, and 0.1% dextrose in phosphate buffered saline (PBS, all chemicals from Sigma Chemical Co.).

PRS (phenol red solution): must be used within 1 hr

aug. PBS	98 parts
phenol red stock	1 part
MRPO stock	1 part

phenol red stock: 0.1g phenol red/10 ml distilled water. This solution (0.028M phenol red) is stable ~6 mo. at 4°C.

HPRO (horseradish peroxidase) stock: 5 mg HRPO (type II, salt-free, Sigma #P-8250)/ml sterile PBS. Usually made in 10 ml lots, subsequently divided into 500μl aliquots, stored at -20°C.

PMA solution: 10 mg PMA (phorbol 12-myristate, 13-acetate, Sigma #P-8139) is dissolved in 8.106 ml dimethyl sulfoxide to give a 2mM PMA stock solution. This is divided into small (~500μl) aliquots and stored at -20°C. The PMA stock solution is further diluted in PRS to obtain a final concentration of 10 nM.

5. Intracellular Superoxide Production (NBT Reduction)

a. NBT Reduction (Pyridine Extraction) by Oyster Macrophages

1. Hemolymph samples were withdrawn from the adductor muscle sinus of oysters held for >2 weeks in flow-through estuarine water (15°C, 18 ppt salinity). Hemolymph samples from several oysters were pooled to obtain about 6.5 ml and held on ice to reduce cell clumping and gently mixed to obtain a uniform suspension. Cells in this suspension were quantified in a hemacytometer.

2. Duplicate 2.0 ml aliquots of hemolymph were added to sterile plastic test tubes. One tube received 1 ml of 0.1% nitroblue tetrazolium (NBT) in filter-sterilized (0.45μm) phosphate buffered (0.01M, pH 7.4) sodium chloride (18 ppt), referred to as "estuarine buffer:

(EB), plus 0.2 ml yeast suspension (10 mg/ml EB); this is the stimulated sample. The second tube (the unstimulated sample) received 1 ml of 0.1% NBT in EB plus 0.2 ml EB.

3. Incubate the tubes 60 min at 21°C, and transfer contents to conical glass centrifuge tubes. The plastic tubes are washed 2x with 2 ml IN HCl (total 4 ml) and wash fluid added to the centrifuge tubes. The tubes were mixed vigorously and centrifuges (10 min, 4°C, 1250xg) and the supernatants discarded and replaced by 1.25 ml pyridine. The reduced NBT (blue formazan) was extracted by placing the tubes in a boiling water bath for 30 min, in a chemical fume hood. Tubes were cooled, centrifuged (10 min, 4°C, 525xg) and the supernatants read vs pyridine at 515 nm.

b. Pyridine Extraction Method (Medaka Macrophages)

1. Introduce 1 ml of macrophage suspension (2 x 10^6 cells) into sterile test tubes, centrifuge (10 min., 21°C, 250 xg), discard supernatants, and resuspend cells in xenobiotic solutions in 1 ml aug. L15 (experimentals) or 1 ml aug. L15 (controls). Duplicate experimental and control tubes will be required. Incubate 1-20 hrs at 21°C.
2. Centrifuge tubes (10 min., 21°C, 250 xg) and replace all supernatants with 1.0 ml aug. L15, and resuspend cells.
3. Procedure with experimental (exposed) macrophages

	unstimulated	stimulated	cell-free control
NBT solution	1.0 ml	1.0 ml	1.0 ml
Macrophages (2×10^6)	1.0 ml	1.0 ml	---
Yeast suspension	---	0.2 ml	---
Aug. L15	---	---	1.2 ml
PBS	0.2 ml	---	---

Incubate above preparations 60 min. at 21°C with gentle agitation every 15 min. Add 2 ml IN HCl to each tube and transfer to conical glass centrifuge tubes; add an additional 2 ml IN HCl, mix and transfer this also to the conical glass centrifuge tubes. All cellular material and particulates should be transferred to the glass tubes. Centrifuge (20 min., 21°C, 125 xg), discard supernatant and resuspend the pellet in 1.25 ml pyridine (Sigma # P-4036). Mix well, cover tubes with foil, place in boiling water bath for 30 min. in a chemical fume hood. Cool tubes in hood. Centrifuge (10 min., 21°C, 525 xg) and read O.D. of supernatant at 515 nm vs. a pyridine blank.

4. Procedure with control (unexposed) macrophages: this is carried out exactly as with exposed macrophages (see #3 above).

5. Calculations: subtract the cell-free control values from the nominal unstimulated and stimulated values obtained in #3 and #4 above. This will give the actual NBT reduction values for the unstimulated macrophages and the phagocytically stimulated macrophages. By comparing these sets of values for unexposed cells vs. xenobiotic exposed cells, one can calculate the % inhibition of NBT reduction (O_2^- generation) by resting and/or stimulated cells.

Solutions

Aug L15: 5% fetal bovine serum (Sigma #F4884) and 3% antibiotic-antimycotic solution (Sigma #A9909) in Leibovitz L15 medium (Sigma #L4386).

NBT solution: Concentrated stock solution is 0.2% nitroblue tetrazolium in phosphate buffered saline (PBS); this is filtered (0.45 μm) and stored at 4°C. The NBT solution used in this assay is 0.1% in PBS, made by 1:1 dilution of the stock solution in PBS, and is used within 3 hrs.

Yeast suspension: Dehydrated bakers yeast (Sigma #YSC02) is resuspended in PBS (2g/50 ml), autoclaved, washed 2x with PBS, divided into small aliquots and stored at -20°C. Prior to use, the actual yeast particle numbers in the suspension are determined with a hemacytometer, and the working yeast suspension prepared (4 x 10^7 yeast particles/0.2 ml PBS, to give a ratio of 20 yeast : 1 macrophage in the assay).

c. DMSO Extraction Method (Medaka Macrophages)

1. Prepare tubes and carry out xenobiotic exposures as per #1. in pyridine extraction method (above).
2. Centrifuge tubes (10 min., 21°C, 250 xg) and resuspend exposed and unexposed macrophages as follows:

	unstimulated	stimulated	cell-free control
NBT solution (0.1%)	2 ml	2 ml	2 ml
PMA solution (1μM)	---	0.022 ml	---
Aug. L15	0.2 ml	0.178 ml	0.2 ml
macrophage pellet (2×10^6 cells)	+	+	---

3. Incubate tubes 60 min. at 21°C.

4. Centrifuge (10 min., 21°C, 525 xg), discard supernatant. Add 2 ml 70% MeOH (methyl alcohol), resuspend, centrifuge (10 min., 21°C, 525 xg), discard supernatant. Resuspend vigorously in 1.25 ml KOH/DMSO solution, centrifuge (10 min., 21°C, 525 xg), read O.D. of supernatant at 625 nm vs. KOH/DMSO solution.

5. Calculations: as per #5. in pyridine extraction method (above).

Solutions

Aug. L15: as per pyridine extraction method

NBT solution: as per pyridine extraction method

KOH/DMSO solution: 6 ml 2M potassium hydroxide (KOH) plus 7 ml dimethyl sulfoxide (DMSO, Sigma #D-8779).

PMA solution: 2mM stock solution = 10 mg PMA (phorbol 12-myristate, 13-acetate) in 8.106 ml DMSO, divided into small aliquots, stored at -20°C. Stock is diluted in aug. L15 to yield 1μM PMA solution for use in assay.

6. In Vivo Exposure of Medaka to Pentachlorophenol (NaPCP)

a. Japanese medaka were acclimated for >2 weeks in 100 gallon aquaria in aerated deep-well water (20-22°C). Throughout acclimation and exposure studies the fish were fed brine shrimp nauplii and commercial flaked food daily. All tests were performed on adult fish (6-12 months of age) held in a 12h L: 12m D photoperiod.

b. Exposure was carried out via a static-renewal procedure, with renewal every 7d. Medaka (20-25 per tank) were placed in 10 gallon aquaria containing 0 (control) to 200ppb NaPCP and monitored daily for mortality.

c. Fish were sacrificed at weekly intervals and chemiluminescence (CL) assays carried out (see CL method). Six macrophage samples were analyzed for each NaPCP concentration and exposure time studied. Each cell sample was comprised of macrophages pooled from the pronephros of 3-5 medaka.

RESULTS AND DISCUSSION

1. Immunotoxicity of Cadmium for the Oyster: Effects on Chemiluminescence

The effects of CD exposure on hemocyte CL activity are shown in serum-free medium (Fig. 1) and in the presence of autologous serum (Fig. 2). These data were generated from aliquots of the same cell pool exposed to various Cd concentrations for 3 h prior to CL determinations; the results are representative of those from the cell pools examined (6 pools - serum, 5 pools +serum). In all cases, both control and Cd-exposed hemocytes produced resting luminol-augmented CL in the absence of phagocytic stimuli. The addition of heat-killed yeast particles to untreated hemocytes induced a marked increase in CL activity that peaked after 5-7

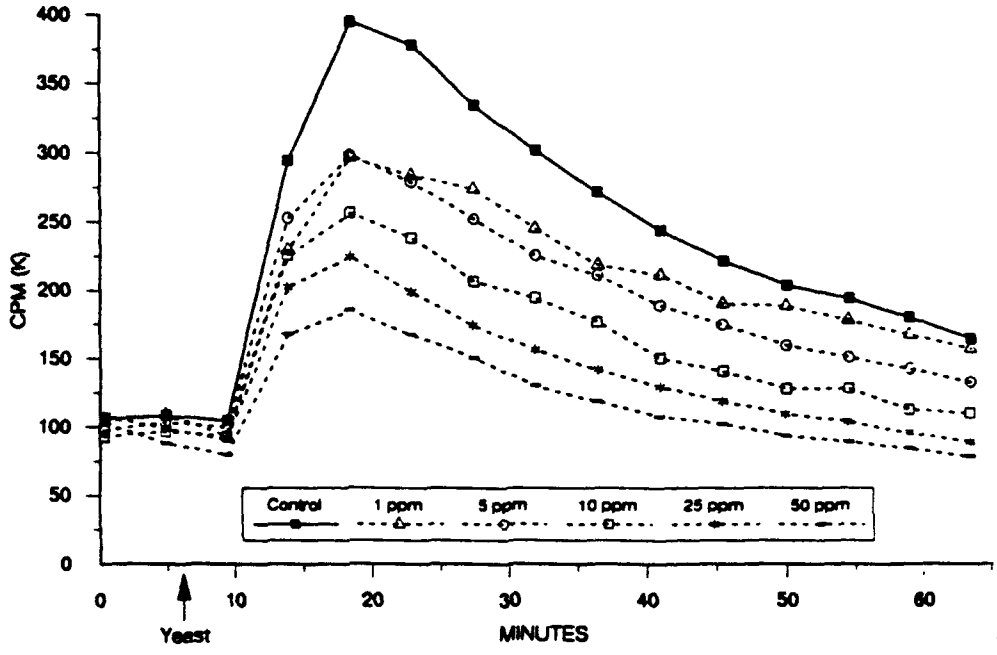


Figure 1. Effects of cadmium exposure on hemocyte chemiluminescence in serum-free medium.

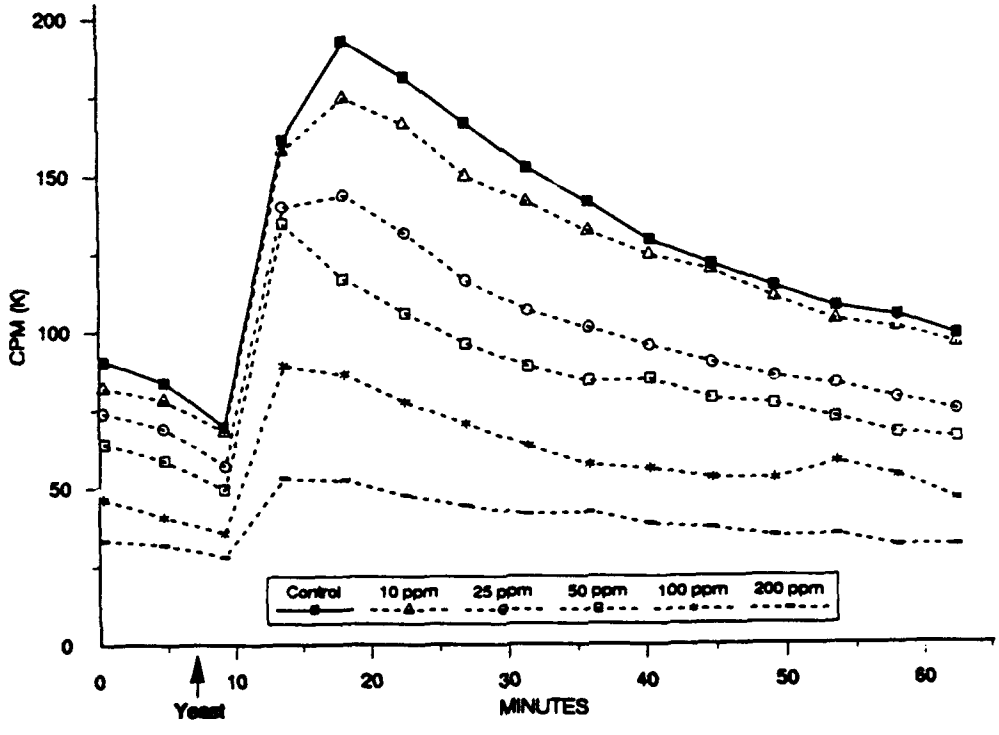


Figure 2. Effects of cadmium exposure on hemocyte chemiluminescence in the presense of serum.

min. This activity gradually decreased with time but remained above background level for more that 60 min. The presence of Cd in the medium consistently produced a dose-dependent suppression of hemocyte-mediated CL; however, higher concentrations were required to produce this effect when oyster serum was included in the medium (Figs. 1 and 2).

The data from the Cd-exposed hemocyte aliquots were expressed as percent inhibition of control CL of an untreated aliquot from the same pool; a summary of these data from six individual pools is presented in Table 1. Arc-sine transformation (Gomez and Gomez 1984) was carried out to normalize the raw percent inhibition data generated from the study. The resultant mean percent inhibition of the various CL parameters induced by 3 h exposure to sublethal cadmium levels are given in the table, along with the upper and lower 99% confidence intervals. These confidence intervals were selected to keep the experiment-wide error rate at ~0.05, using the method of Bonferroni (Tarone 1990). Cadmium concentrations that produced inhibitions with lower 99% confidence intervals $<5\%$ were considered to have no effect on chemiluminescence. The highest cadmium exposure levels used in the serum + and serum - groups were selected based on their lack of 3 h lethality, by the trypan blue exclusion assay. Concentrations of 10, 25, and 50 ppm were run on both groups to enable direct evaluation of serum effects on CL.

Interpretation of the data in Table 1 leads to the following conclusions. Although a trend of increasing inhibition of basal CL with increasing Cd concentrations is seen, examination of the lower 99% confidence interval values suggests that there is no meaningful inhibition at any Cd level tested, with or without serum in the medium. However, Cd does suppress peak CL in the absence of serum ($\geq$10 ppm) and in the presence of serum ($\geq$100 ppm). A similar effect was seen in the case of total CL, where dose-dependent suppression was induced at $\geq$2 ppm (in serum

Table 1. Percent inhibition of hemocyte chemiluminescence (CL) by cadmium

	Serum-Free Media				Serum-Containing Media		
	Cd ppm	Mean Percent Inhibition	Lower 99% CI	Upper 99% CI	Mean Percent Inhibition	Lower 99% CI	Upper 99% CI
Basal CL	1	2.6	- 2.3	20.8			
	2	5.9	0.7	16.1			
	10	5.7	- 0.7	28.4	1.9	- 2.3	17.0
	25	13.1	1.0	35.8	4.8	- 1.3	27.8
	50	13.7	1.2	36.3	7.8	- 0.4	34.7
	100				21.6	- 5.8	87.5
	200				38.3	1.0	89.3
Peak CL	1	16.0	0.3	48.1			
	2	23.0	2.3	56.1			
	10	36.3	8.5	70.5	17.0	- 1.9	69.7
	25	60.4	31.8	85.9	39.8	24.2	56.5
	50	66.3	43.9	85.4	34.4	0.9	84.1
	100				78.3	23.6	99.1
	200				92.5	62.8	99.0
Total CL	1	15.4	0.0	52.8			
	2	26.2	5.9	54.4			
	10	43.0	5.3	86.7	21.9	2.7	52.4
	25	69.1	38.5	92.5	34.8	24.5	45.8
	50	75.8	52.6	92.9	40.2	14.3	69.4
	100				74.4	51.9	91.5
	200				90.9	67.9	100.0

Basal CL = chemiluminescence produced by hemocytes prior to phagocytic stimulation.
Peak CL = maximal chemiluminescence recorded after phagocytic stimulation of the cells.
Total CL = the area under the curve of chemiluminescent activity induced by phagocytosis.

free media) and ≥25 ppm (in serum-containing medium). These data indicate that more cadmium was required to produce levels of inhibition of peak or total chemiluminescence comparable to those seen in the absence of serum. Other pieces of supporting evidence included the 3 h EC_{50} (effective concentration of Cd that produced 50% inhibition of peak CL after 3 h exposure, calculated by linear regression) data; these were 73 ppm in the presence of serum, and 21 ppm in the absence of serum. The cadmium effects on CL could not be attributed to lethality because, even at the highest concentrations tested, the average reductions in viability were minimal (3.2% at 50 ppm Cd, - serum; 1.5% at 200 ppm Cd, + serum).

The data were analyzed by ANOVA to determine the statistical significance of the changes in CL parameters observed following exposure to cadmium and the apparent protective effect of serum in the medium (Table 2). If significance is set at $p \leq 0.05$, it can be concluded that cadmium produced marked inhibition of phagocytically induced peak and total chemiluminescence, but caused no significant reduction in unstimulated, resting CL. Using the same criterion, serum in the medium protected against the CL suppression by cadmium, regardless of which CL parameter was considered. As indicated in Table 2, there were no interactive effects (synergy, etc.) of the CL responses to cadmium and serum. The protective effect of oyster serum in these in vitro assays was predictable. No doubt extracellular Cd was bound to serum proteins and possibly to other ligands such as metallothioneins. The data reemphasized the fact that caution is needed in interpreting in vitro toxicity determinations. In this case, inclusion of autologous serum to mimic physiological conditions caused apparent reduced sensitivity, probably by reducing the actual dose of the toxicant available to the hemocytes.

Table 2. Percent inhibition of hemocyte chemiluminescence (CL) by cadmium: Direct comparison of 10, 25, and 50 ppm and effect of autologous serum.

	p Values, Data Analyzed by ANOVA		
	Cd Effect	Serum Effect	CD * Serum Interaction
Basal CL	0.1387	0.0205	0.9205
Peak CL	0.0078	0.0202	0.7635
Total CL	0.0052	0.0193	0.6235

Some preliminary attempts were made to determine the Cd concentration in the hemocytes under the conditions of the experiment. Exposure to Cd was carried out following the protocols previously reported, the cells washed thoroughly to remove unincorporated Cd and atomic absorption analyses of the cells was performed by the Riverside Clinical Laboratories (Newport News, VA). Cells were exposed to 200 ppm Cd (+ serum) or 50 ppm Cd (- serum) for 3 h, treatments already shown to strongly inhibit the CL response. In this study, both treatments produced ~50% reduction in peak CL and total CL, somewhat less inhibition than would be predicted based on Table 1, but substantial. Hemocytes exposed to 200 ppm Cd (+serum) contained an average of 15.0 μg Cd/mg protein, those exposed to 50 ppm Cd (- serum) contained an average of 15.8 μg Cd/mg protein. Based on these limited data, it appeared that modulation of CL was primarily a function of cellular Cd level.

In this regard, we had previously attempted to study the effects on subsequent hemocyte CL after two weeks of in vivo exposure of oysters to water borne Cd (Oliver and Anderson

unpublished). Several Cd levels were tried, including 0.25 ppm (nominal) which was selected as the highest experimental dose. Cadmium toxicity, as manifested by decreased condition index (Roesijadi and Klerks 1989) and reduced shell growth (Shuster and Pringle 1969), starts to become evident at ~0.20 ppm. The mean Cd concentration in hemocytes from the 0.25 ppm group was 2.2 μg Cd/mg protein; Cd concentrations in hemocytes from unexposed oysters were several orders of magnitude lower. The effects on CL responses of hemocytes collected from oysters after in vivo exposure to a range of Cd concentration $\leq$0.25 ppm were variable and showed no significant dose dependency.

However, as reported in this paper, in vitro exposure of oyster hemocytes to sublethal concentrations of Cd can suppress CL, suggesting impairment of a cell-mediated cytotoxic mechanism, such as bactericidal activity. Such evidence implicates Cd as a potential immunotoxicant, by extension of the criteria developed for mammals (Tam and Hinsdill 1990). Our inability to show CL inhibition in the whole-animal exposure studies possibility may be explained by the fact that the requisite hemocyte Cd levels (indicated by the in vitro results) were not attained.

Few reports exist concerning the effects of metals, or other environmental contaminants, on the CL response of bivalve hemocytes. Copper ($\geq$4 ppm) was shown to inhibit phorbol myristate acetate-induced oyster hemocyte CL, whereas cadmium, aluminum and zinc were reported to slightly enhance CL at $\leq$160 ppm, but suppressed CL at a higher concentration of 320 ppm (Larson et al. 1989). It appears that our current method can more sensitively monitor CL modulation by Cd; this could result from differences in the composition of the media, length of exposure, and nature of the respiratory burst stimulus. As part of a study of tributyltin (TBT)

on defense-related activities of oyster hemocytes, Fisher et al. (1990) showed that 0.4-400 ppb TBT produced a dose dependent inhibition of zymosan-induced CL activity. Our data confirm and extend these observations on the utility of hemocyte CL modulation as a method to identify environmental xenobiotics with potential immunotoxicity for bivalve mollusks. Furthermore, CL provides a sensitive means of more fully understanding the details of protective mechanisms available to invertebrate blood cells.

2. Superoxide Generation by Oyster Hemocytes as Measured by Nitroblue Tetrazolium Reduction

NBT Reduction by Hemocyte Monolayers

Initially, NBT formazan production was observed by microscopic examination of individual hemocytes. Whole hemolymph samples were placed on ethanol cleaned coverslips and allowed to adhere for 30 min at room temperature in a moist chamber. Serum and nonadherent cells were gently washed off the resultant cell monolayers with (0.45 μm) filter-sterilized ambient estuarine water (EW). NBT vials (Sigma Diagnostics) were reconstituted with 1 ml of 10% NaCl to give a final solution of phosphate-buffered 18 ppt NaCl (to emulate the salinity of estuarine water) containing 1 mg/ml NBT. This solution was diluted with EW or a suspension of 0.2 mg/ml autoclaved yeast cells (1 part NBT solution:2 parts EW + particles). Unstimulated cell preparations were exposed to NBT in the absence of particles; stimulated samples were incubated with both NBT and yeast. Hemocyte monolayers were incubated at room temperature for 2 hr with the above described working NBT solutions, and the NBT was then washed off and replaced with EW. The coverslips were inverted, placed on slides, and examined as wet mounts. Viewing

with bright-field optics was preferable to phase contrast because none of the blue shading and artifacts seen with phase contrast were encountered.

Superoxide anion production by the hemocytes was indicated by the intracellular deposition of blue formazan (reduced NBT). The appearance of these cells is described without photomicrographs because of the difficulty of distinguishing blue-stained organelles and membrane-associated structures from their unstained counterparts in black and white prints. Unstimulated or stimulated preparations contained no blue-stained cellular components in the absence of NBT. Characteristically, the monolayer preparations contained numerous cells with distinct cytoplasmic granules (granulocytes), as well as cells with few or no granules (hyalinocytes). In unstimulated or stimulated preparations exposed to NBT, the hyalinocytes rarely contained blue deposits, blue-staining cytoplasm, or cytoplasmic elements. Therefore, the following description of NBT reduction refers to events taking place in the granulocytic hemocytes.

NBT reduction was seen in some of the unstimulated granulocytes. In these cells, a positive response typically involved a diffuse pale blue staining of the cytoplasm with more intense deposits along the ruffled portion of the plasma membrane. Such cells often appeared larger than typical granulocytes due to more extensive spreading. Intracellular particulate formazan deposits and stained granules were rarely seen in cells that did not contain phagocytosed yeast particles. The ingestion of yeast particles was often accompanied by intense NBT reduction; particulate formazan deposits were frequently seen in the cytoplasm and especially in the vacuoles surrounding the phagocytosed particles. Hemocytes with positive reactions associated with ingested yeast cells usually also had pale blue cytoplasm.

In order to assess NBT reduction by this method, at least 300 hemocytes were examined from unstimulated and stimulated preparations from five individual oysters. Cells with light blue-stained cytoplasm constituted 43.7 + 9.9% of the unstimulated and 42.3 + 12.0% of the stimulated populations. In the stimulated groups, 13.7 + 4.5% of these cells also contained ingested particles surrounded by intense blue deposits; in other words, about one-third of the cells with bluish cytoplasm contained phagocytosis-associated NBT reduction.

Quantitative NBT Reduction

In order to look for possible seasonal effects on NBT reduction, oysters from the same area (Broomes Island) were collected at various times from January 1990 to October 1990. Water temperature and salinity were recorded at the time, and the NBT assays were performed within 2 days of collection. About 200 oysters collected from the Broomes Island site at the beginning of the study were maintained in the laboratory in flow-through tanks supplied with unfiltered, ambient estuarine water. These lab-held oysters were assayed for NBT-reducing ability at about the same times as the field samples were collected and analyzed. The purpose of this portion of the work was to determine if NBT data gathered from laboratory-maintained oysters were representative of those obtained from the field.

This assay provided highly reproducible data when run on aliquots from the same hemolymph pool. However, it became apparent that the response showed great variability from pool to pool, even in replicates taken at the same time. Cell numbers and/or cellular protein concentrations were recorded for every hemocyte pool. When NBT reduction was expressed on a per hemocyte basis the observed variation was not reduced. The inability to correct resting or

stimulated NBT reduction for variations in cell numbers or cell protein concentration suggests a high level of natural variation in this activity in oysters. The reason(s) that hemocytes from oysters collected under identical conditions from the same site showed such widely different capacities for NBT reduction is unknown. Possibly this reflects genetic heterogeneity, differences in the microenvironment of the animals, differences in health and disease, differences in reproductive status, etc.

In order to minimize cell clumping and to provide the most natural physiological conditions for phagocytosis, the assays were carried out with a standard (2 ml) aliquot of whole hemolymph, containing about 2 x 10^6 cells. The numbers of yeast particles provided to the hemocytes were experimentally determined to exceed the phagocytic potential of the cells; therefore, variations in the cell:particle ratio resulting from differences in cell counts per milliliter of hemolymph had no effect on the recorded extent of NBT reduction.

Inclusion of 600-3000 U/ml of superoxide dismutase in the medium consistently produced a decreased level of NBT reduction by both resting and phagocytically stimulated cells. The levels were reduced from ~20 to 50% in a dose-dependent manner; this degree of inhibition by SOD is typical of that seen in other leukocyte studies.

Data from oysters sampled at various times during a 10-month period are presented in Table 3. There were no statistically significant differences in hemocyte NBT reduction between samples from field or lab-held oysters collected at identical times over the course of this study. Apparent phagocytically induced increments in NBT reduction were seen in all groups, but these were not significant in any one group. Both stimulated and unstimulated levels of NBT reduction tended to increase with elevation of the ambient water temperature, but were independent of

salinity in the relatively narrow range experienced (~ 10-14‰). These trends were statistically significant when data from the relatively cool periods (January and April; 2 to 13°C) were compared to those from the warm periods (July and October; 21 to 29°C) in Table 4. The levels of NBT reduction achieved by both resting and stimulated hemocytes were significantly increased in samples obtained during the warmer months ($P < 0.001$). Hemocytes from oysters at 21-29°C responded to phagocytic stimulation with a significant rise in NBT reduction ($P < 0.05$), but this effect was not seen in the 2-13°C group.

Table 3. NBT Reduction by Oyster Hemocytes

Field-collected oysters				Laboratory-held oysters			
Date and water conditions	Unstimulated	Stimulated	Δ OD	Date and water conditions	Unstimulated	Stimulated	Δ OD
1/17/90 1.8°C, 10.8 ‰	68 ± 32	73 ± 32	5 ± 17	12/31/89 4°Cm 13,2 ‰	93 ± 62	109 ± 68	16 ± 18
4/13/90 11°C, 9.9 ‰	190 ± 55	232 ± 49	42 ± 12	4/16/90 13°C, 10.8 ‰	130 ± 108	152 ± 96	22 ± 13
7/18/90 28°C, 12 ‰	324 ± 191	429 ± 171	105 ± 45	7/24/90 29°C, 12.3 ‰	361 ± 125	463 ± 111	102 ± 35
				10/16/90 21°C, 14 ‰	329 ± 137	438 ± 132	109 ± 18

Note: Data are OD units $x10^3$ at 515 nm, mean ± SD, n=5. Laboratory-held oysters were collected 8/23/89 from the Broomes Island area and maintained in flow-through tanks supplied with unfiltered estuarine water. Field-collected oysters were assayed for NBT reduction within 2 days of collection.

Table 4. Effect of Ambient Temperature on NBT Reduction by Oyster Hemocytes

Temperature range	Unstimulated	Stimulated	Δ OD
2 - 13°C	120 ± 64	142 ± 61	22 ± 15
21 - 29°C	238 ± 144	443 ± 131	105 ± 33

Note: Data are OD units $x10^3$ at 515 nm, mean ± SD, n = 20 for 2-13°C, n = 15 for 21-29°C.

The purpose of these studies was to further support the contention that superoxide anion generation may be a general property of phagocytic leukocytes in invertebrates, as well as vertebrates, by examining NBT reduction by C. *virginica* hemocytes. A positive reaction was observed microscopically and morphological details of NBT formazan deposition in the cells are presented below, as are attempts to quantify the percentages of phagocytes involved. However, comments on the relative amounts of formazan present in cell populations by visual observation under various conditions are subjective at best. This is especially relevant because changes in the overall level of NBT reduction in a given cell population seem to reflect changes in the intensity of the reaction in particular cells, not involvement of different numbers of cells. Therefore, the cytological results are considered qualitative and cannot be directly compared to results from the quantitative NBT reduction assays. The cytological observations provided the basis for adapting the clinical quantitative NBT assay for use with oyster cells; the accepted interpretation of the results of these assays is probably valid for phagocytes in general.

Our studies of NBT reduction by oyster granulocytes can be compared to those of human leukocytes carried out by Gifford and Malawista (1970,1972). Upon exposure to NBT, about 40% of adherent human and oyster granulocytes became filled with a diffuse blue stain and often showed a more intense reaction at their periphery. These cells often were atypically enlarged by extensive spreading and have been called "formazan" cells in the mammalian literature. As in the case with human cells, the percentage of formazan cells in adherent oyster cell monolayers was not increased by phagocytosis. As previously described, NBT formazan cells can arise independently of phagocytic stimulation and about one-third of them will also take up available yeast particles. Ingestion of yeast triggers additional formazan deposition in phagosomes and in

other areas of the cytoplasm. An enlarged, flattened morphology and concomitant cellular degeneration can be seen in formazan cells from both species. Although formazan cells appear abnormal, Gifford and Malawista (1972) found that their formation did not result from loss of membrane integrity and that cell degeneration followed (and did not precede) production of intracellular formazan. NBT can gain access to the cytoplasm during phagocytosis, but in the absence of phagocytosis, NBT may enter the hemocyte during its attempt to "phagocytize" the glass substrate to which it adheres.

Human blood cells with defective leukocytic antibacterial capacity (CGD or chronic granulomatous disease of childhood) cannot reduce NBT and cannot produce formazan cells, either in suspension culture or when adhered to a substrate. All preparations of oyster hemocytes examined showed NBT-reducing ability whether in suspension (via the quantitative NBT reduction assay) or after adherence (in hemocyte monolayers). This does not eliminate the possibility that rare genetically based immunological defects similar to CGD may exist in oysters; only about 30 individuals were examined via the monolayer method and the work with the quantitative NBT assay was done with pooled samples from about 150 oysters.

Dikkeboom and co-workers (1987, 1988) described NBT reduction by hemocyte monolayers derived from several snail species. Their findings with regard to SOD-sensitive NBT reduction are similar to these with oysters and those of Gifford and Malawista with human cells. They reported faint background staining of unstimulated hemocytes (formazan cells?) and intense dark blue formazan deposits inside phagosomes around the ingested zymosan particles.

NBT reduction has previously been observed microscopically in several molluscan species, but in this report an attempt was made to quantify it. Despite variability within groups, statistical

analysis of the data permitted several conclusions to be drawn. Hemocytes withdrawn from oysters at any time of year showed NBT-reducing ability without stimulation, and this activity was markedly elevated during the warmest portion of the year. In addition, phagocytosis increased total NBT reduction by hemocytes from oyster held above $-20°C$, but this effect was not seen in cells collected below $\sim 13°C$.

These results support the idea that the ability to generate cytotoxic oxygen radicals is a general property of leukocytes. The exact physiological role of hemocyte-produced superoxide anion in mollusks has not been established, but it is probably involved in internal defense responses. The simple quantitative assay for O_2^- described herein may prove useful in assessing a mechanism of immunocompetency in animals collected under various environmental conditions. Modulation of hemocytic NBT reduction might also have application as a biomarker of stress induced by sublethal exposure to xenobiotics.

3. In Vitro Inhibition of Medaka Phagocyte Chemiluminescence by Pentachlorophenol.

Approximately 95% of medaka pronephros cells remained viable after 1 h of *in vitro* maintenance, ~ 90% survived 20 h in culture (as determined by trypan blue exclusion). Exposure to PCP produced dose-dependent lethality that was particularly evident in the 20 h cultures (Fig.3). Under the conditions of this study, it was estimated by regression analysis that the 1 h $LC_{50} > 75$ ppm, and the 20 h $LC_{50} \sim 28$ ppm.

The kinetics of the phagocytically-induced CL response were different when elicited after 20h of culture, as compared to 1 h of culture. Representative kinetic curves are shown in Fig. 4. Phagocytes from any given cell pool, when stimulated after 1 h, produced a steadily rising

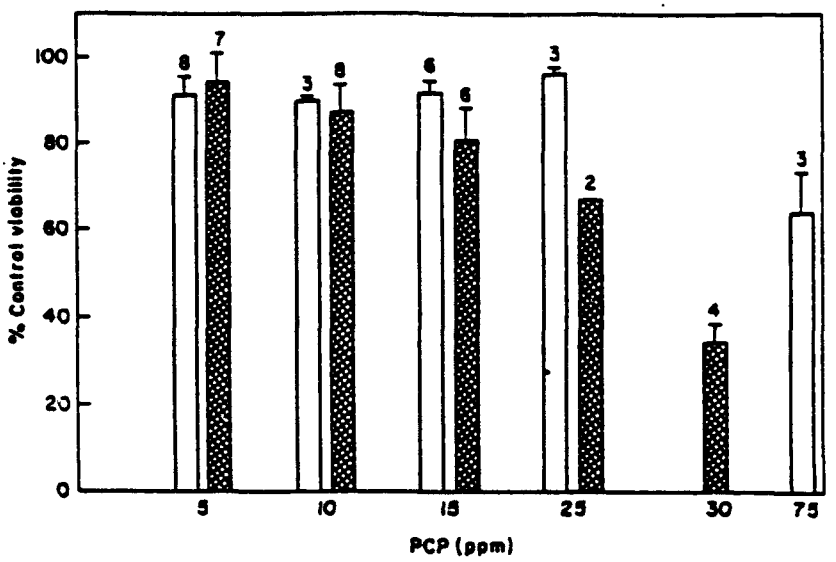


Figure 3. Viability of medaka pronephros cells after incubation with analytical grade PCP for 1 h (open bars) and 20 h (cross-hatched bars). Values represent mean percent of unexposed control viability ± S.D.; *N* for each group is noted above the error bar. The viabilities of the cells in the unexposed control groups were 98·8 ± 2·0(12) after 1 h, and 89·6 ± 2·

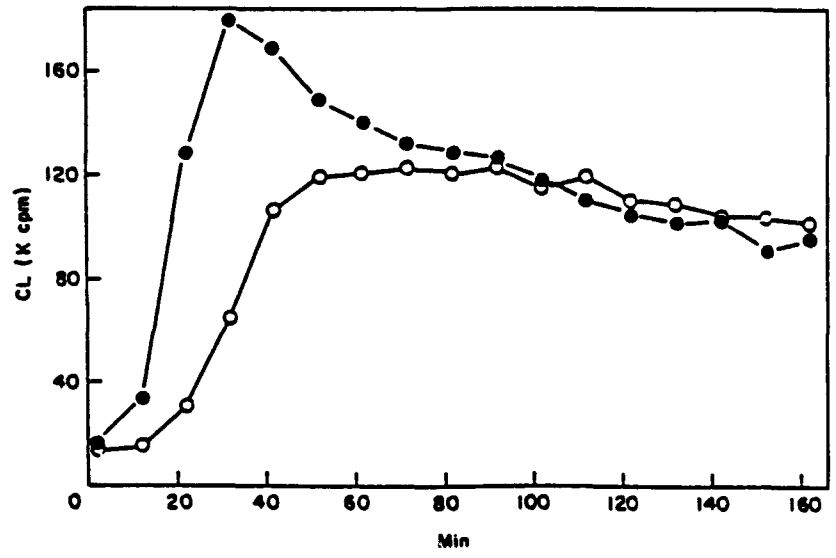


Figure 4. The chemiluminescent response induced by phagocytic stimulation in two aliquots from the same pool of unexposed pronephros cells after 1 h of culture (open circles) and after 20 h of culture (closed circles).

CL response that tended to plateau and remain relatively constant for at least several hours. In contrast, 20 h samples responded with a more rapid CL activity increment, produced a clearly defined peak, and then the activity gradually decayed. On many occasions, the 20 h CL peak and total CL value exceeded the comparable 1 h values; however, this was not always observed.

The effects of A-PCP exposure on CL by medaka phagocytes are presented graphically in Figs 5 and 6. Figure 5 shows the result of 1 h incubation in the presence of 5-15 ppm A-PCP. A clear, dose-dependent reduction in phagocytically-induced CL is seen with almost total inhibition of CL at 20 ppm (Table 5). The typical 1 h incremental CL response and leveling off is seen in control; A-PCP exposure shifts the curves downward without significantly altering their shape. The ability of cells exposed to various A-PCP doses for 20 h to respond to phagocytic stimulation (Fig. 6) is also markedly compromised.

Data from representative phagocyte pools are illustrated in Figs 3 and 4; and the mean CL values from all cell pools examined in this study are summarized in Table 5. The CL parameters given are expressed as percent inhibition of untreated control cells after 1 and 20 h and include unstimulated CL (background CL produced by cells in the absence of added yeast cells), stimulated CL peak activity (peak value minus background), and total stimulated CL activity (area under the curve minus background level). All three CL parameters were inhibited in a dose-dependent fashion by 1 or 20 h exposure to 5-20 ppm A-PCP. As might be expected, inhibition was more pronounced in the 20 h groups, as was PCP-related loss of viability. However, even at the higher concentrations, the observed lethality could not totally account for the elevated levels of CL suppression. It appeared that the PCP effect on CL could easily be

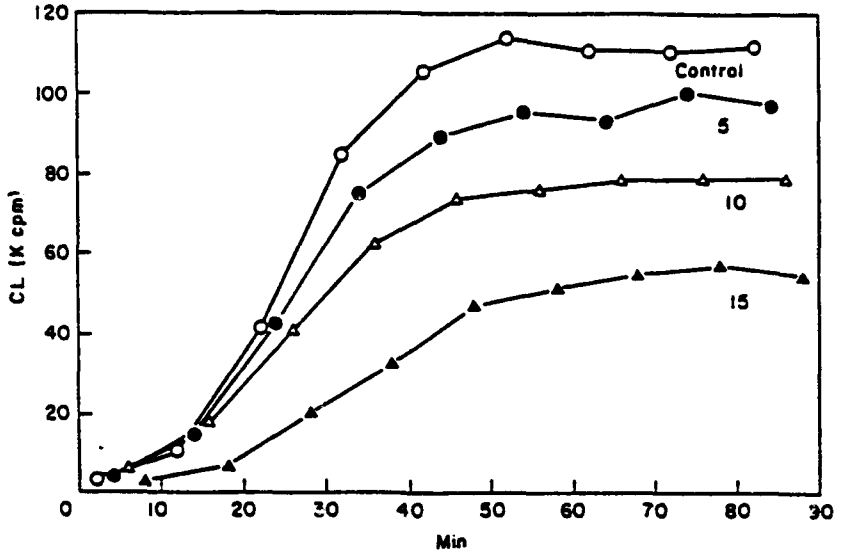


Figure 5. Typical family of curves showing the effects of chemiluminescence of exposure to various concentrations of analytical grade PCP for 1 h; curves are labeled with the nominal PCP levels (ppm) in the medium.

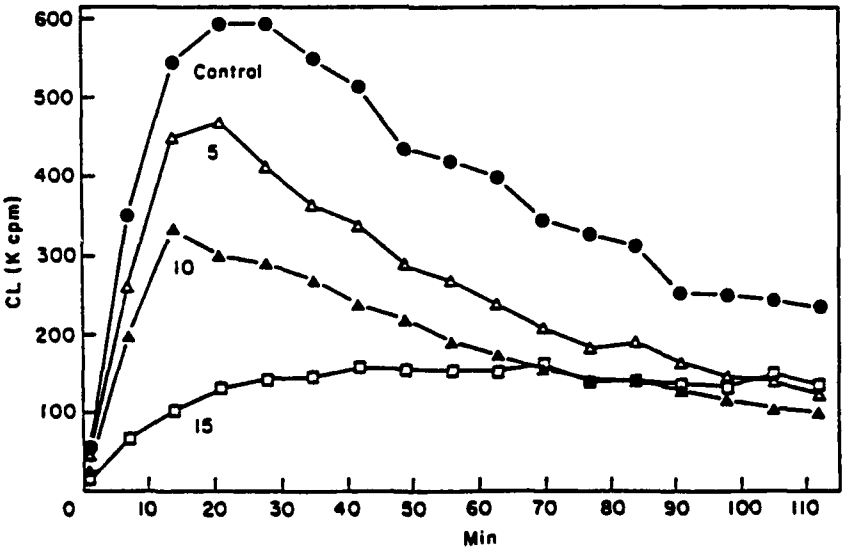


Figure 6. Representative graphs of phagocytically induced chemiluminescence by cells which had been previously incubated for 20 h with 0-15 ppm analytical grade PCP.

Table 5. Inhibition of pronephros cell CL by exposure to analytical grade PCP*

NaPCP (ppm)	Viability		Unstimulated CL		Stimulated CL peak		Stimulated total CL	
	1 h	20 h	1 h	20 h	1 h‡	20 h	1 h	20 h
5.0	>95	>95	21.0 ± 15.0(7)†	24.7 ± 14.4(6)	11.9 ± 6.0	16.7 ± 8.3	9.2 ± 3.3	16.4 ± 10.2
10.0	>95	~94	14.6 ± 8.9(5)	56.6 ± 18.8(6)	29.7 ± 1.9	37.0 ± 8.3	23.5 ± 12.6	31.0 ± 15.5
15.0	>95	~81	31.8 ± 21.2(7)	68.6 ± 11.5(3)	42.6 ± 9.6	61.0 ± 9.0	46.8 ± 13.3	53.1 ± 15.2
20.0	>95	~65	93.0 (2)	97.0 ± 0.9(3)	93.6	100.0	94.4	96.8 ± 2.0

* Values are expressed as mean percent inhibition compared to unexposed control cell values ± S.D.

† Group sizes in parentheses are the same in corresponding entries in the following columns of CL parameters.

‡ After 1 h culture, phagocytic stimulation produces a sustained plateau of CL activity rather than a well-defined peak; therefore, in this column the maximal CL level achieved after PCP exposure is compared to the maximal CL level of the unexposed control.

measured after a 1 h exposure. The 1 h test was shown to have about the same sensitivity as the 20 h test without any measurable effects on cell viability.

Because PCP-mediated inhibition of stimulated peak and stimulated total CL responses were virtually identical (Table 5), only total CL data are presented in Table 6 which compares the effect of A-PCP to that of T-PCP. After 20 h exposure, T-PCP apparently caused more CL suppression than did A-PCP at > 5 ppm; this difference was significant at 15 ppm.

The PCP effect on CL was reversible, as shown in Table 7. In this experiment, pronephros cells were incubated for 1 h in 25 ppm A-PCP, a treatment that resulted in >95% suppression of CL but no significant reduction in cell viability. The exposed cells were washed and resuspended in PCP-free medium. CL activity was restored by approximately 70% in about 4 h, and had returned to that of unexposed cells by about 24 h.

As is the case for mammalian macrophages, fish macrophages (comprising 50 60% of the pronephros cells) are essential accessory and effector cells in the immune system and changes in their responsiveness could have widespread repercussions in the host (Luster *et* al., 1982). Measurement of chemiluminescence serves as an important primary screening assay to assess immunomodulatory activities of environmental toxicants on mammalian macrophages (Tam & Hinsdill, 1990) and seems to find similar application to studies of fish immunotoxicology. In these studies, exposure to either A-PCP or T-PCP was shown to result in CL suppression in medaka leucocytes at sublethal concentrations. This effect was both dose-dependent and reversible. Previous studies with various species of fish have described inhibition of CL by metals, organometallics and polycyclic aromatic hydrocarbons (Elsasser et al., 1986; Warinner et al., 1988; Wishkovsky et al., 1989).

Table 6. Effect of 20 h exposure to analytical or technical grade PCP on CL response to phagocytic stimulation.

PCP (ppm)	Analytical grade	Technical grade	P
1·0	0 (2)	0 (2)	NS
5·0	16·4±10·2(6)	15·2±4·5(5)	NS
10·0	31·0±15·5(6)	42·5±7·8(5)	NS
15·0	53·1±15·2(3)	84·0±6·9(4)	<0·005
20·0	96·8± 2·0(3)	100·0 (2)	NS

Data are expressed as mean percent inhibition of PCP-exposed cells compared to data from unexposed controls ± S.D. (*N*).

Table 7. Recovery of CL response following removal of pronephros cells from 25 ppm PCP-containing medium

Hours in PCP-free medium	Total CL (% of control)	Cell viability
0	<5·0	>95
3·0- 5·5	71·3±19·1(3)	~95
21·0-26·0	94·4± 5·6(3)	~90

Data are expressed as mean percent of unexposed control cells ± S.D. (*N*).

The immunotoxic effects of PCP have been studied in birds and mammals. Prescott et al. (1982) fed chickens a purified grade of PCP, that contained 300 ng g^{-1} heptachlorodibenzo-p-dioxin (HpCDD) and 800 ng g^{-1} octachlorodibenzo-pdioxin (OCDD) but no tetrachloro- or hexachloro-dibenzo-p-dioxins (TCDD, HCDD). After 8 weeks the humoral and cellular immune responses were evaluated. Chickens receiving 2500 ppm PCP showed decreased lectin-induced lymphoproliferation, lower white blood cell counts, a decreased humoral response to bovine serum albumin, but normal levels of IgM and IgG and normal humoral responses to sheep erythrocytes and Newcastle disease virus. Exposure of mice to T-PCP resulted in enhancement of Moloney sarcoma virus (MSV)-induced tumor susceptibility, inhibition of cytotoxic T cell activity, a slight increase in resistance to encephalomyocarditis virus (EMCV), and increased phagocytosis by peritoneal macrophages (Kerkvliet et al., 1982b). Macrophages play a primary role in resistance to EMCV infection, while T cells are involved in the regression of MSV-induced sarcomas. No significant changes in immune function were induced by A-PCP administration. Mice fed T-PCP, but not A-PCP, showed depressed humoral immune responses to both T-dependent and T-independent antigenic stimulation (Kerkvliet et al., 1982a). Rats fed 97% pure PCP had decreased antibody titers, decreased delayed-type hypersensitivity and increased phagocytic activity by peritoneal macrophages (Exon & Koller, 1983). In order to examine in greater depth the sensitivity of T cells, natural killer cells and macrophages to T-PCP, Kerkvliet et al. (1985a) studied their activities in mice following 8 weeks of dietary exposure. The only significant alteration in the parameters tested was a reduced lymphoproliferative response in mixed lymphocyte culture, but not in the response to mitogens. It was concluded that T cells, NK cells and macrophages were relatively resistant to T-PCP, in

contrast to its marked effects on the humoral immune response. Several contaminant fractions and purified isomers from T-PCP were analyzed for their humoral immunosuppressive effects (Kerkvliet et al., 1985b). The 1,2,3,6,7,8-hexachlorodioxin, 1,2,3,4,6,7,8-heptochloro-dioxin, and 1,2,3,4,6,7,8-heptachlorofuran were all immunosuppressive. This and other evidence was presented for the role of toxic Ah-interactive dioxin and furan contaminants in T-PCP mediated immunotoxicity.

In light of the above, it is interesting that the immunosuppressive effect of A-PCP on medaka phagocyte CL is virtually equivalent to that of T-PCP. The exact chemical composition of the T-PCP (Aldrich Chemical Company, Inc., lot 1907MH) was not known; however, the manufacturer determined that it was ~ 97% pure PCP, although 86% purity was indicated on the label. Because of the variation in percent and identities of specific contaminants, it may be difficult to compare immunotoxic activities of T-PCPs used in various studies. We report that 15 ppm T-PCP caused significantly more CL suppression than 15 ppm A-PCP. This effect was not seen at lower PCP concentrations, while higher levels of either T- or A-PCP essentially abolished CL. Whether the slightly enhanced immunosuppressive effect of T-PCP was caused by contamination with dioxins and/or furans has yet to be determined.

It was evident that A-PCP (PCP sodium salt hydrate, > 99% pure, lot 03512JM, Aldrich Chemical Company, Inc.) caused dose-dependent suppression of CL by medaka phagocytes, despite its reported lack of effects on humoral or cellular immune parameters in mammals. Previously, A-PCP (Aldrich Chemical Company, Inc., lot 032487CC) has been reported to cause decreased resistance to bacterial infection (Anderson et al., 1981) in the bivalve M. *mercenaria,* probably as a consequence of impaired-hemocyte-mediated antibacterial capacity (Anderson,

1988). In this context, it is interesting to note that 2,4-dinitrophenol, another uncoupler of oxidative phosphorylation, has been reported to inhibit superoxide release and CL by rat and rabbit alveolar macrophages (Miles et al., 1977; Castranova et al., 1987).

4. Quantitative NBT reduction by medaka macrophages (via DMSO/KOH extraction), as influenced by exposure to PCP.

In 5 attempts, exposure of medaka macrophages to 5-30 ppm PCP for 1 h produced no significant effect on either cell viability or NBT reduction. However, effects on both parameters were seen after 20 h exposure to PCP.

Viability of macrophages after 20 h of PCP exposure are shown in Table 8; clearly concentrations ≥20 ppm produced high lethality. In all cases there was a basal (unstimulated) level of NBT reduction by the macrophages in both control and PCP-exposed groups. Stimulation of the control cells by 1μM phorbol myristate acetate (PMA) resulted in increased NBT reduction in every case: $\Delta OD = 0.088 \pm 0.059$ (7). Exposure of the cells to PCP produced an apparent dose-dependent inhibition of PMA-induced NBT reduction (Table 9). However, sublethal effects (≤ 15 ppm) produced such variable results (ranges: 5 ppm = 0-39%, 10 ppm = 0-100%) that this assay was not continued as a measure of in vitro PCP immunotoxicity. The CL assay provided data with much greater sensitivity to sublethal PCP concentrations (see Table 5).

Table 8. Viability of medaka macrophages after 20 hour exposure to PCP.

PCP (ppm)					
Control	5	10	15	20	30
97.4 ± 1.8 (5)	96.2 (1)	93.7 ± 3.6 (5)	89.5 ± 10.2 (2)	45.5 ± 1.5 (2)	18.5 (1)

Table 9. Percent inhibition of NBT reduction by 20 h exposure to PCP (by DMSO/KOH extraction)

PCP (ppm)				
5	10	15	20	30
22.8 ± 18.9 (4)	53.2 ± 47.6 (6)	27.7 (1)	86.5 ± 1.1 (2)	100.0 (1)

5. Quantitative NBT reduction by medaka macrophages (via pyridine extraction), as influenced by PCP exposure.

As per the previous section, inhibition of NBT reduction after 20 h exposure to PCP was measured, but by the more traditional pyridine extraction method (Baehner and Nathan, 1968). This seemed to give slightly more sensitivity at 15 ppm, but gave equally variable results at lower concentrations (Table 10) as did the DMSO/KOH method of extraction of the formazan. However, working with hot pyridine is potentially more hazardous than DMSO/KOH.

Table 10. Percent inhibition of NBT reduction by 20 h exposure to PCP (by the pyridine extraction method).

PCP (ppm)					
5	10	15	20	25	30
13.6 ± 30.4 (5)	42.0 ± 30.1 (6)	99.6 ± 10 (5)	100.0 (2)	100.0 (1)	100.0 (2)

6. Extracellular superoxide release by medaka cells: the effect of 20 h PCP exposure.

Attempts were made in three experiments (covering different ranges of PCP concentrations) to detect modulation of O_2^- production via the cytochrome c assay. Preliminary results showed no dose-dependency and uninterpretable data, such as only 45.5% inhibition at 50 ppm (a dose known to be lethal to >82% of the macrophages (Table 8). This data is presented in Table 11. This assay was not continued in light of the greater sensitivity and reproducibility of CL as a measure of the effects of xenobiotics on ROI production (Table 5).

Table 11. Percent inhibition of O_2^- production by 20 h exposure to PCP (via cytochrome c) by medaka macrophages.

PCP (ppm)					
5	15	20	25	30	50
29.6 (1)	15.5 (2)	0 (1)	10.0 (2)	8.1 (1)	45.5 (1)

7. In Vivo Exposure of Medaka to Pentachlorophenol

Pentachlorophenol concentrations ≤ 75 ppb produced about the same mortality as that seen in the control groups; however, 200 ppb PCP was rapidly lethal (Table 12).

Table 12. Cumulative Mortality of NaPCP (based on 3 studies)

Day	PCP Concentration (ppb)					
	0	25	50	75	100	200
1						
2						1
3					1	7
4						3
5	1					
6			1			
7						
8					1	
9		1				
10						
11						
12						
13						
14					1	
Mortality 14d	1/65	1/45	1/45	0/20	3/65	11/20*

Note: * 11/20 dead by day 4, all sacrificed

The peak CL response of macrophages was significantly inhibited ($P<0.001$) by 14d exposure of medaka to 100 ppb NaPCP in the first experiment. In an attempt to duplicate this effect and to establish evidence of dose-dependency, a second experiment was carried out with 75, 100, and 200 ppb. As noted on Table 13, 200 ppb was rapidly lethal to the fish; however, 3 cell pools of macrophages from fish remaining alive on day 3 were analyzed. Unfortunately, all experimental groups in experiment two were not different from control CL, although all conditions were as identical to experiment one as possible. A third in vivo experiment was run in order to resolve this discrepancy concerning the effect of 100 ppb; 25 and 50 ppb effects were also measured. After one week none of the experimental peak CLs differed significantly from the controls, however, once again 100 ppb exposure for 14 days produced significant ($P<0.025$) inhibition in peak CL (Table 13).

Table 13. Peak Chemiluminescence Values (10^3 x cpm $\pm$ SD, n = 6/group) of Medaka Macrophages Exposed to Pentachlorophenol (in vivo exposure)

Experiment	NaPCP concentration (ppb)					
	0	25	50	75	100	200
1 (14 days)	476 ± 64	405 ± 187	375 ± 164		147 ± 46[1]	
2 (14 days)	238 ± 48			357 ± 109	311 ± 245	162 ± 110[2]
3 (7 days)	236 ± 84	282 ± 74	258 ± 97		321 ± 46	
(14 days)	190 ± 25	221 ± 50	148 ± 36		141 ± 29[3]	

[1] Significantly different from control ($P<0.001$)
[2] Data from only 3 cell pools on day 3, PCP lethal at this concentration
[3] Significantly different from control ($P<0.025$)

Thus it appeared that sublethal PCP exposure of medaka in vivo could cause an immunotoxic effect in pronephros cells similar to that produced by in vitro exposure of the cells to the same chemical stressor. This conclusion is based on results from two of three preliminary studies with a single xenobiotic, clearly more work in this area will be required. Nevertheless, these results were encouraging and suggest that immunotoxic responses to contaminated water samples may indeed be measured by quantifying CL modulation in fish phagocytic blood cells.

CONCLUSIONS

Among other sublethal toxicological endpoints, immunosuppression can be produced in mammals by chemical exposure. Modulation of immune parameters in lower animals by exposure to environmental contaminants has received much less study. This project was an attempt to quantify aspects of this phenomenon in several aquatic species: the oyster (*Crassostrea virginica*) and a fish (the medaka, *Oryzias latipes*). These and other metazoan species utilize macrophages as a first line of internal defense against infectious diseases; the cells are also important in antigen processing and presentation to lymphocytes, as well as other immunoregulatory functions. Macrophages represent a conserved cell type; therefore, it is likely that immunotoxicological data obtained from macrophages from any species may have potential for interspecies extrapolation. The aspect of macrophage function selected for this study was reactive oxygen intermediate (ROI) production, a function intimately associated with the cells' defensive capacity via its role in antimicrobial activity. Chemically-induced reduction in ROI generation by macrophages is a measure of immunosuppression, which is probably indicative of increased susceptibility to disease.

Work carried out with funding from this grant led to the following conclusions.

1. Both the oyster (*C. virginica*) and the medaka (*O. latipes*) contain macrophages that produce reactive oxygen intermediates (ROIs). ROI activity was measured by several in vitro assays that detect superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (.OH) and mixtures of these ROIs. Macrophages from both oysters and medaka produced ROIs in short-term culture without the addition of respiratory burst stimuli; however, the addition of such stimuli generally resulted in increased ROI generation. This increase in activity was seen after phagocytosis of particulates (zymosan or yeast cells) or after contact with cell surface receptor-mediated stimuli (phorbol myristate acetate).

2. Cadmium, a common environmental contaminant, was shown to produce a dose-dependent suppression of oyster macrophage chemiluminescence (CL). Macrophage CL is an accurate means of quantifying ROI production, hence immunocompetency. These results were produced by in vitro exposure of the macrophages and were taken as an indication of potential Cd immunotoxicity. Subsequent in vivo exposure of oysters to Cd produced no consistent CL suppression in the macrophage cell population. It was determined that immuno-suppressive intracellular Cd concentrations reached in vitro were not reached in our in vivo study. It was likely that the protective effect of serum proteins by Cd sequestration noted in our in vitro work explained, in part, the in vivo results.

3. Oyster hemocytes were shown to produce superoxide (O_2^-) by nitroblue tetrazolium (NBT) reduction, both microscopically and quantitatively following extraction. NBT reduction was recorded in unstimulated macrophages withdrawn from animals throughout the year, but significant induction by phagocytosis was seen only in cells collected from oysters collected from warm environments (21° - 29°C) vs. those collected in cooler (2° - 13°C) times of the year. The apparent seasonality of ROI production by oyster macrophages had not been previously determined.

4. Quantitative NBT reduction by medaka macrophages consistently showed levels over resting when stimulated by phorbol myristate acetate (PMA). Incubation with pentachlorophenol (PCP) produced dose-dependent reduction in cell viability; however, while the apparent reduction in NBT reduction tended to increase with PCP concentration, the effect was not entirely dose-dependent and was highly variable. Another O_2^- assay using ferricytochrome C to measure ROI inhibition produced by PCP exposure gave no better results.

5. Medaka macrophages produced a strong phagocytically-induced, luminol-dependent CL response. The magnitude of this CL response was strongly influenced by the presence of sublethal PCP concentrations in vitro. In this way the immunosuppressive effects of pure PCP (analytical grade, A-PCP) on medaka CL was quantified. The effect was attributed to the activity of A-PCP as an uncoupler of oxidative phosphorylation. Technical grade PCP (T-PCP) is contaminated with various dioxins and dibenzofurans

which are thought to exert their toxic activities via interaction with the Ah receptor. T-PCP was only slightly more immunosuppressive than A-PCP, as measured by inhibition of medaka macrophage CL. The presence of the Ah receptor in fish macrophages has not been determined; however, it has been reported in fish hepatocytes.

6. In vivo PCP exposure studies were carried out with medaka. It was found that 62/65 (~95%) of the fish survived 14 day exposure to 100 ppb PCP. In two of three in vivo exposure studies, the CL response of macrophages withdrawn from fish exposed to 100 ppb PCP was significantly inhibited; in all studies, lower concentrations (25, 50 75 ppb for 14 days) produced no significant effect.

7. These preliminary studies suggest that (in medaka) a chemical (PCP) identified as a potential immunotoxicant via an in vitro screening test (luminol-augmented CL), was in fact capable of suppressing ROI production in fish exposed to sublethal in vivo concentrations. It is recommended that additional studies along these lines be undertaken to determine the generality of these observations using other environmental xenobiotics.

CHRONOLOGICAL BIBLIOGRAPHY OF PUBLICATIONS OF STUDIES SUPPORTED IN PART BY THIS GRANT

I. Papers (peer-reviewed, open literature)

A. Published

1. Anderson, R.S., Oliver, L.M. and Brubacher, L.L. 1992. Superoxide anion generation by *Crassostrea virginica* hemocytes as measured by nitroblue tetrazolium reduction. *J. Invertebr. Pathol. 59:303-307.*

2. Anderson, R.S., Oliver, L.M. and Jacobs, D. 1992. Immunotoxicity of cadmium for the eastern oyster (*Crassostrea virginica* [Gmelin, 1791]): effects on hemocyte chemiluminescence. *J. Shellfish Res. 11:31-35.*

3. Anderson, R.S. and Brubacher, L.L. 1992. *In vitro* inhibition of medaka phagocyte chemiluminescence by pentachlorophenol. *Fish and Shellfish Immunol. 2:299-310.*

4. Anderson, R.S., Mora, L.M. and Brubacher, L.L. 1992. Sensitivity of oyster hemocyte chemiluminescence to antibiotic-antimycotic agents. *J. Invertebr. Pathol. 60:317-318.*

5. Anderson, R.S. and Brubacher, L.L. 1993. Pentachlorophenol inhibits production of reactive oxygen intermediates by medaka phagocytic blood cells. *Mar. Environ. Res. 35:125-129.*

6. Anderson, R.S. 1993. Modulation of nonspecific immunity by environmental stressors. In: *Pathobiology of Marine and Estuarine Organisms*, J. Couch and J. Forney (eds.). CRC Press, Inc., Boca Raton, FL. pp. 483-510.

B. In Press

1. Anderson, R.S. Modulation of blood cell mediated oxyradical production in aquatic species: implications and applications. In: *Molecular and Biological Approaches to Aquatic Toxicology*, G.K. Ostrander and D.C. Malins, eds. CRC Press, Boca Raton, FL.

II. Technical Reports

A. In Press

1. Anderson, R.S. and Brubacher, L.L. Development of immunotoxicological assays for the Japanese medaka: Generation of cytoxic oxygen radicals by phagocytic cells using a bivalve model system. *Compendium of the FY 1988 and FY 1989 Research Reviews for the Research Methods Branch.* USABRDL, Frederick, MD. pp. 120-132.

2. Anderson, R.S. and Brubacher, L.L. Immunotoxicology in the medaka: Chemical modulation of macrophage chemiluminescence. *Compendium of the FY 1990 and FY 1991 Research Reviews for the Research Methods Branch.* USABRDL, Frederick, MD.

3. Anderson, R.S. Modulation of oxyradical generation by medaka phagocytes following exposure to environmental chemicals. *Compendium of the FY 1990 and FY 1991 Research Reviews for the Research Methods Branch,* USABRDL, Frederick, MD.

III. Abstracts of Presentations at Scientific Meetings (locations in parentheses)

1. Anderson, R.S. 1989. Effects of pollutant-exposure on hemolymph-mediated immune function. *J. Shellfish Res. 8:435-436.*

   (Symposium on Effects of Anthropogenic Inputs on Bivalves, at National Shellfisheries Association annual meeting, Williamsburg, VA)

2. Anderson, R.S. 1990. Immunomodulation by exposure of shellfish and finfish to xenobiotics: implications for altered resistance/susceptibility to disease. *Abstracts of the EPA Gulf Breeze Symposium on Marine and Estuarine Disease Research, p.33.*

3. Anderson, R.S. and Oliver, L.M. 1991. Suppression of oyster hemocyte chemiluminescence by cadmium. *Abstracts of the 24th Annual Meeting of the Society for Invertebrate Pathology,* pp.95-96.

4. Roszell, L.E. and Anderson, R.S. 1991. Influence of pentachlorophenol (PCP) on the chemiluminescent response of *Fundulus heteroclitus* phagocytes. *Amer. Zool. 31:130A.*

   (Presented at the American Society of Zoologists annual meeting at Atlanta, GA)

5. Anderson, R.S. and Brubacher, L.L. 1991. Reduced production of reactive oxygen intermediates by pronephros phagocytes after exposure to pentachlorophenol. *Abstracts of the 6th International Symposium on Responses of Marine Organisms to Pollutants, p.2.*

6. Roszell, L.E. and Anderson, R.S. 1992. Effects of pentachlorophenol on the chemiluminescent response of phagocytes from two estuarine species. *Toxicologist 12:392.*

   (Presented at the Society of Toxicologists annual meeting, Seattle, WA)

7. Anderson, R.S. 1992. Oxyradical production by oyster hemocytes: Modulation by environmental chemicals and disease. *Abstracts of the 25th Annual Meeting of the Society for Invertebrate Pathology, p.85.*

   (Held at the University of Heidelberg, Germany).

REFERENCES

Adema, C.M., Harris, R.A., and van Deutekom-Mulder, E.C. 1992. A comparative study of hemocytes from 6 different snails: morphology and functional aspects. J. Invertebr. Pathol., 59:24.

Adema, C.M., van Deutekom-Mulder, E.C., van der Knapp, W.P.W., Meuleman, E.A., and Sminia, T. 1991. Generation of oxygen radicals in hemocytes of the snail *Lymnaea stagnalis* in relation to the rate of phagocytosis. Dev. Comp. Immunol., 15:17.

Adema, C.M., Van der Knapp, W.P.W., and Sminia, T. 1991. Molluscan hemocyte-mediated cytotoxicity: the role of reactive oxygen intermediates. Rev. Aquat. Sci., 4:201.

Anderson, R. S. 1988. Effects of pollutant exposure on bactericidal activity of *Mercenaria mercenaria* hemolymph, Extended Abstracts, Division of Environmental Chemistry. Amer. Chem. Soc. Nat. Meeting, 28:248.

Anderson, R.S. and Brubacher, L.L. 1993. Inhibition by pentachlorophenol of production of reactive-oxygen intermediates by medaka phagocytic blood cells. Mar. Environ. Res., 35:125.

Anderson, R.S., Mora, L.M., and Thomson, S.A. 1993. Copper inhibits oxyradical production by oyster macrophages. Toxicologist, 13:183.

Anderson, R.S., and Brubacher, L.L. 1992. In vitro inhibition of medaka phagocyte chemiluminescence by pentachlorophenol. Fish and Shellfish Immunol., 2:229.

Anderson, R.S., Mora, L.M., and Thomson, S.A. 1992. Exposure of oyster macrophages to particulate brass suppresses luminol-augmented chemiluminescence. Toxicologist, 12:391.

Anderson, R.S., Oliver, L.M., and Jacobs, D. 1992. Immunotoxicity of cadmium for the eastern oyster (*Crassostrea virginica*) [Gmelin, 1791]: effects on hemocyte chemiluminescence. J. Shellfish Res., 11:31.

Anderson, R.S., Giam, C.S., Ray, L.E., and Tripp, M.R. 1981. Effects of environmental pollutants on immunological competency of the clam, *Mercenaria mercenaria*: impaired bacterial clearance. Aquat. Toxicol., 1:187.

Bachère, E., Hervio, D., and Mailhe, E. 1991. Luminol-dependent chemiluminescence by hemocytes of two marine bivalves, *Ostrea edulis* and *Crassostrea gigas*. Dis. Aquat. Orig., 11:173.

Badwey, J.A. and Karnovsky, M.L. 1980. Active oxygen species and the functions of phagocytic leukocytes. Ann. Rev. Biochem., 49:695.

Baehner, R.L. and Nathan, D.G. 1968. Quantitative nitroblue tetra-zolium test in chronic granulomatous disease. New Engl. J. Med. 178: 971.

Beaman, L. and Beaman, B.L. 1984. The role of oxygen and its derivatives in microbial pathogenesis and host defense. Ann. Rev. Microbiol., 38:27.

Castranova, V., Lee, P., Ma, J.Y.C., Weber, K.C., Pailes, W.H., and Miles, P.R. 1987. Chemiluminescence from macrophages and monocytes, In: Cellular Chemiluminescence (Van Dyke, K., and Castranova, V., eds.) CRC Press, Boca Raton. P 3.

Cheng, T.C. 1976. Aspects of substrate utilization and energy requirement during molluscan phagocytosis. J. Invertebr. Pathol., 27:263.

Dikkeboom, R., van der Knapp, W.P.W., van den Bovenkamp, W., Tinjnagel, J.M.G.H., and Bayne, C.J. 1988. The production of toxic oxygen metabolites by haemocytes of different snail species. Dev. Comp. Immunol., 12:509.

Dikkeboom, R., Tijnagle, J.M.G.H., Mulder, E.C., and van der Knapp, W.P.W. 1987. Hemocytes of the pond snail *Lymnea stagnalis* generate reactive forms of oxygen. J. Invertebr. Pathol., 49:321.

Dikkeboom, R., Mulder, E.C., Tijnagel, J.M.G.H., and Smina, T. 1985. Phagocytic blood cells of the pond snail *Lymnaea stagnalis* generate active forms of oxygen. Eur. J. Cell. Biol. Suppl., 11:12.

Elsasser, M.S., Roberson, B.S., and Hetrick, F.M. 1986. Effects of metals on the chemiluminescent response of rainbow trout (*Salmo gairdneri*) phagocytes. Vet. Immunol. Immunopathol., 12:243.

Exon, J.H. and Koller, L.D. 1983. Effects of chlorinated phenols on immunity in rats. J. of Immunopharmacology, 5:131.

Fisher, W.S., Wishkovsky, A., and Chu, F-L.E. 1990. Effects of tributyltin on defense-related activities of oyster hemocytes. Arch. Environ. Contam. Toxicol., 19:354.

Fridovich, I. 1988. The biology of oxygen radicals: general concepts. In: Oxygen Radicals and Tissue Injury (B. Halliwil, ed.) FASEB for the Upjohn Company, Bethesda, MD. P. 105.

Gifford, R.H., and Malawista, S.E. 1972. The nitroblue tetrazolium reaction in human franulocytes adherent to a surface. Yale J. Biol. Med., 45:119.

Gifford, R.H., and Malawista, S.E. 1970. A simple rapid micromethod for detecting chronic granulomatous disease of childhood. J. Lab. Clin. Med., 75:511.

Gomez, K.A. and A.A. Gomez. 1984. In: Statistical Procedures for Agricultural Research. Wiley & Sons, New York. P. 306.

Hetrick, F.M., Knittel, M.D., and Fryer, J.L. 1979. Increased susceptibility of rainbow trout to infectious hematopoietic necrosis virus after exposure to copper. Appl. Environ. Microbiol., 37:198.

Horan, T.D., English, D., and McPherson, T.A. 1982. Association of neutrophil chemiluminescence with microbicidal activity. Clin. Immunol. Immunopathol., 22:259.

Kerkvliet, N.I., Brauner, J.A., and Baecher-Steppan, L. 1985a. Effects of dietary technical pentachlorophenol exposure on T cell, macrophage and natural killer cell activity in C57B1/6 mice. Journal of Immunopharmacology, 7:239.

Kerkvliet, N.I., Brauner, J.A., and Matlock, J.P. 1985b. Humoral immunotoxicity of polychlorinated diphenyl ethers, phenoxyphenols, dioxins and furans present as contaminants of technical grade pentachlorophenol. Toxicology, 36:307.

Kerkvliet, N.I., Baecher-Steppan, L., Claycomb, A.T., Craig, A.M., and Sheggeby, G.G. 1982a. Immunotoxicity of technical pentachlorophenol (PCP-T): depressed humoral immune responses to T-dependent and T-independent antigen stimulation in PCP-T exposed mice. Fundamental and Applied Toxicology, 2:90.

Kerkvliet, N.I., Baecher-Steppan, L., and Schmitz, J. 1982b. Immunotoxicity of pentachlorophenol (PCP): increased susceptibility to tumor growth in adult mice fed technical PCP-contaminated diets. Toxicology and Applied Pharmacology, 62:55.

Klebanoff, S.J. 1968. Myeloperoxidase-halide-hydrogen peroxide antibacterial system, J. Bacteriol., 95:2131.

Knittel, M.D. 1981. Susceptibility of steelhead trout *Salmo gairdneri* Richardson to redmouth infection *Yersenia ruckeri* following exposure to copper. J. Fish Dis., 4:33.

Larson, K.G., Roberson, B.S., and Hetrick, F.M. 1989. Effect of environmental pollutants on the chemiluminescence of hemocytes from the American oyster *Crassostrea virginica*. Dis. Aquat. Org., 6:131.

LeGall, G., Bachère, E., and Mailhe, E. 1991. Chemiluminescence analysis of the activity of *Pecten maximus* hemocytes stimulated with zymosan and host-specific Rickettsiales-like organisms. Dis. Aquat. Org., 11:181.

Luster, M.I., Dean, J.H., and Moore, J.A. 1982. Evaluation of immune functions in toxicology. In: Principles and Methods of Toxicology (A.W. Hayes, ed.) Raven Press, New York. P. 561.

Miles, P.R., Lee, P., Trush, M.A., and Van Dyke, K. 1977. Chemiluminescence associated with phagocytosis of foreign particles in rabbit alveolar macrophages. Life Sci., 20:165.

Nakamura, N., Mori, K., Inooka, S., and Nomura, T. 1985. In vitro production of hydrogen peroxide by the amoebocytes of the scallop, *Patinopecten yessoensis* (Jay). Dev. Comp. Immunol., 9:407.

National Research Council. 1992. Animal models for use in detecting immunotoxic potential and determining mechanisms of action. In: Biologic Markers in Immunotoxicology. National Academy Press, Washington, D.C. P. 83.

Rice, C.D., and Weeks, B.A. 1990. The influence of in vitro exposure to tributyltin on reactive oxygen formation in oyster toadfish macrophages. Arch. Environ. Contam. Toxicol., 19:854.

Rice, C.D., and Weeks, B.A. 1989. Influence of tributyltin on in vitro activation of oyster toadfish macrophages. J. Aquat. Animal Health, 1:62.

Roesijadi, G., and Klerks, P.L. 1989. Kinetic analysis of cadmium binding to metallothionein and other intracellular ligands in oyster gills. J. Exp. Zool., 251:1.

Roszell, L.E., and Anderson, R.S. 1992. Effects of pentachlorophenol on the chemiluminescent response of phagocytes from two estuarine species. Toxicologist, 12:392.

Scott, A.L., Rogers, W.A., and Klesius, P.H. 1985. Chemiluminescence by peripheral blood phagocytes from channel catfish: Function of opsonin and temperature. Dev. Comp. Immunol., 9:241.

Scott, A.L., and Klesuis, P.H. 1981. Chemiluminescence: a novel analysis of phagocytosis in fish, In: Developments in Biological Standardization (Anderson, D.P. and Hennessen, W., eds.), S. Krager, Basel 49:245.

Secombes, C.J., Cross, A.R., Sharp, G.J.E., and Garcia, R. 1992. NADPH oxidase-like activity in rainbow trout *Oncorhynchus mykiss* (Walbaum) macrophages. Dev. Comp. Immunol., 16:405.

Secombes, C.J., Fletcher, T.C., O'Flynn, J.A., Costello, M.J., Stagg, R., and Houlihan, D.F. 1991. Immunocompetence as a measure of the biological effects of sewage sludge pollution in fish. Comp. Biochem. Physiol., 100C:133.

Shuster, C.N., Jr., and Pringle, B.H. 1969. Trace metal accumulation by the American eastern oyster, *Crassostrea virginica*. Proc. Natl. Shellfish Assoc., 59:91.

Stave, J.W., Roberson, B.S., and Hetrick, F. 1984. Factors affecting the chemiluminescent response of fish phagocytes. J. Fish Biol., 25:197.

Stave, J.W., Roberson, B.S., and Hetrick, F.M. 1983. Chemiluminescence of phagocytic cells isolated from the pronephros of striped bass. Dev. Comp. Immunol., 7:269.

Tam, P.E., and Hinsdill, R.D. 1990. Screening for immunomodulators: effects of xenobiotics on macrophage chemiluminescence in vitro. Fund. Appl. Toxicol., 4:542.

Warinner, J.E., Mathews, E.S., and Weeks, B.A. 1988. Preliminary investigations of the chemiluminescent response in normal and pollutant-exposed fish. Mar. Environ. Res., 24:281.

Welsh, W.D. 1980. Correlation between measurements of the luminol-dependent chemiluminescence response and bacterial susceptibility to phagocytosis. Infect. Immun., 30:370.

Wishkovsky, A. 1988. Chemiluminescence: an advanced tool for measuring phagocytosis, In: Disease Processes in Marine Bivalve Molluscs, (Fisher, W.S. ed.). Amer. Fish. Soc. Spec. Publ. 18:292.

Wishkovsky, A., Mathews, E.S., and Weeks, B.A. 1989. Effect of tributyltin on the chemiluminescent response of phagocytes from three species of estuarine fish. Arch. Environ. Contam. Toxicol., 18:826.

Tarone, R.E. 1990. A modified Bonerroni method for discrete data. Biometrics, 46:515.